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Highlights

- A new formulation of NBPT, a known urease inhibitor, with cyclodextrin was prepared
- The complex CD/NBPT was fully characterized
- The stability of NBPT and that of CD/NBPT in acidic media were studied by NMR
- IC_{50} of the CD/NBPT complex was determined

From algal polysaccharides to cyclodextrins to stabilize a urease inhibitor

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Abstract: *N*-Butyl-phosphorotriamide (NBPT) is a fertilizer widely used for its urease inhibiting properties. Nevertheless, formulations currently commercialized are complex and do not avoid severe decrease of activity due to the low stability of the bioactive compound under acidic conditions. According to its structure, NPBT was thought to be able to interact with both polar additives, by its phosphoramidate function, and hydrophobic ones, through its alkyl chain. In this context, and in order to simplify formulations of this bioactive compound, a panel of natural polysaccharides was studied, including starch, β -(1,3)-glucans, carraghenans and alginates. We also used cyclodextrins, characterized the most stable

inclusion complex with α -cyclodextrin and evaluated the stability of NBPT thus protected against hydrolysis under acidic conditions.

Keywords: NBPT; Carraghenans; Alginates; (1,3)-Glucans; Starch; Cyclodextrins

1. Introduction

The small molecule of urea is the source of two major scientific discoveries. Not only it is the first molecule chemically synthesized by Wöhler in 1828, but the study of its biodegradation allowed the first crystallization of an enzyme, the Jack Bean urease, in 1926 by Sumner (Sumner, 1926), who was subsequently awarded by the Nobel Prize for this breakthrough in 1946. Meantime, urea has become an important component of many fertilizers. However, as only 30% of the available nitrogen is absorbed by plants, improving the efficiency of the nitrogen use in agriculture generally requires adding inhibitors acting on nitrification and/or ureases. The application of the latter significantly reduces ammonia volatilization, as well as damages to seedlings. Plants are also able to assimilate nitrogen as urea, and urease inhibitors promote this type of nutrition, which is also beneficial for plant growth (Trenkel, 2010). Finally, the use of such compounds able to increase the lifetime of urea in soils is not exclusive to agriculture, but find applications in dairy farming where they can significantly decrease nitrogen emissions due to urine and manure (Leinker, Reinhardt-Hanisch, Hartung & von Borell, 2005; Zaman, Nguyen, Blennerhassett & Quin, 2008).

Numerous studies have led to the marketing of a large panel of molecules having anti-ureasic activities [for instance hydroquinone, phosphotriamide compounds (Domínguez et al., 2008; Font et al., 2008; Trenkel, 2010)], or able to influence nitrification phenomena [ammonium thiosulfate, thiourea, or more complex products, i.e. pyrimidinic, triazolic, thiadiazolic derivatives, dicyandiamide (DCD) (Nelson & Huber, 1992; Trenkel, 2010)], both contributing

to improve nitrogen absorption by plants. In addition to the range of such bioactive molecules, many formulations are proposed. Inhibitors may be available as pure compounds or directly incorporated into fertilizers. They can also be formulated in liquid or solid phases. This allows matching a wide range of treatments to soil characteristics and infrastructure of the users. For instance, commercial forms of DCD are an excellent example of adaptability, since it is possible to obtain this single molecule or in combination with other inhibitors of nitrification possibly mixed with urea, or even incorporated into fertilizers containing ammonium salts and many other nutrients. Similarly, *N*-butyl-thiophosphotriamide (NBPT) can be purchased in a liquid formulation (Agrotain), but also in a solid one containing DCD, or in solid granules with urea and DCD. Consequently, commercially available inhibitors stabilize nitrogen for several days to several weeks.

The development of new compounds and/or formulations is part of an integrated approach to farming, keeping in mind that control of the global impact on environment is a priority. However, decades of use of urease inhibitors, and of basic researches, highlighted the complexity of using them efficiently. Among influencing parameters are the quality of soils (pH, nature of the organic matter and possibility to find colloids, porosity, biodiversity linked with numerous microorganisms), abiotic factors (temperature, water quality), and own properties of the inhibitors (volatility, hydrophilic/hydrophobic balance, stability and mobility in soils) (Subbarao et al., 2006). In this context, we have initiated a program and first proposed to covalently connect known nitrification inhibitors to mono- and disaccharides, especially in order to increase their ecocompatibility (Pro et al., 2012). Herein, we propose another approach lying on the opportunity to formulate the known urease inhibitor NBPT (Fig. 1) with natural polysaccharides and derivatives thereof. It is recognized that NBPT has already inhibition properties ($IC_{50} = 100$ nM), but this thiophosphoramidate is readily converted

into its *O*-counterpart NBPTO that is the real inhibitor in soils ($IC_{50} = 1 \text{ nM}$). However, two major limitations are generally encountered with this inhibitor patented in 1985 (Kole, Swerdloff, Rogic, Hendrickson & Van Der Puy, 1985): the first one is its hydrophobicity that requires more or less complex formulations, and the second but more limiting one relies on its low stability, and so loss of activity, in acidic media (Domínguez et al., 2008). The ability of carbohydrates to adopt many conformations, and/or to interact with their aqueous environment (basic vs. acidic, presence of salts) (Cui, 2005), offers great opportunities to develop new formulations.

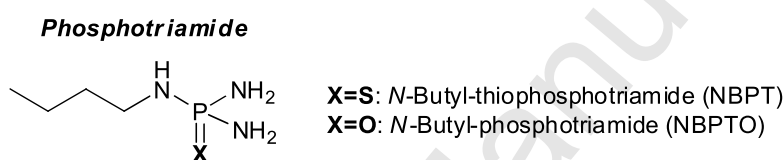


Fig.1. Structure of the studied urease inhibitor NBPT.

2. Material and methods

2.1. Materials

Polysaccharides used in this study are commercially available. NBPT was provided by Timac Agro International (Dinard) and was further purified by recrystallisation from water. ^1H , ^{13}C , ^{31}P NMR spectra were recorded with a Brüker ARX 400 spectrometer at 400 MHz, 100 MHz, and 162 MHz for ^1H , ^{13}C and ^{31}P , respectively. Chemical shifts are given in δ units (ppm). Coupling constants J were calculated in Hertz (Hz). Abbreviations were used to precise signal multiplicity: s (singlet), d (doublet), t (triplet), m (multiplet), dd (double doublet)... The HRMS were measured at the Centre Regional de Mesures Physiques de l'Ouest (CRMPO, Université de Rennes 1) with a MS/MS ZabSpec TOF Micromass using *m*-nitrobenzyl alcohol as a matrix and accelerated caesium ions for ionization.

2.2. Degradation tests in acidic media by ^{31}P NMR

The necessary amount of formulation for having 10 mg of NBPT was mixed with 250 μL water in NMR tubes (blank solution consisted in 40 mg Agrotain in 250 μL of water). The solution was heated until complete dissolution of NBPT. 250 μL of acidic buffer solution ($\text{CH}_3\text{COOH}/\text{CH}_3\text{COONa}$, 500 mM, pH 5.5) was added. Then, a fused capillary containing a invariant H_3PO_4 solution in D_2O was inserted, and the degradation of NBPT was monitored by ^{31}P NMR. The degradation of NBPT was deduced from the decrease of the signal at 60.5 ppm (corresponding to the NBPT) compared to the constant -0.3 ppm peak's area (corresponding to the H_3PO_4).

2.3. Formulations with cyclodextrin $\text{CD}\alpha$ and determination of the inclusion complex thanks to the Job's method

A solution of α -cyclodextrin ($\text{CD}\alpha$) in water (10 mM) and a solution of NBPT in methanol (10 mM) were prepared. Different ratio of both solutions were mixed together in test tubes (from 0:10 to 10:0), for a total volume of 1 mL, before freeze-drying. The resulting solid was dissolved into 1 mL of D_2O , and analyzed by ^1H NMR. For each ratio of NBPT (r , Eq. 1) and for each proton (i) of NBPT, the variation of chemical shift ($\Delta\delta_r^i$) was calculated as (Eq. 1):

$$r = [\text{NBPT}]/([\text{NBPT}] + [\text{CD}\alpha]) \quad (\text{Eq. 1})$$

$$\Delta\delta_r^i = \delta_r^i - \delta_1^i \quad (\text{Eq. 2})$$

For each ratio of $\text{CD}\alpha$ ($1-r$) and for each proton (j) of $\text{CD}\alpha$, the variation of chemical shift $\Delta\delta_{1-r}^j$ was calculated as (Eq. 3):

$$\Delta\delta_{1-r}^j = \delta_{1-r}^j - \delta_0^j \quad (\text{Eq. 3})$$

Then $r.\Delta\delta_r^i$ and $(1-r).\Delta\delta_{1-r}^j$ were plotted against r . Resulting curves went through a maximum, at the r value corresponding to the ratio of the inclusion complex.

2.4. Determination of the association constant

The association constant was obtained according to the method of Benesi-Hildebrand (Benesi & Hildebrand, 1949). A solution of NBPT in methanol (5 mM) and a solution of CD- α in water (125 mM) were prepared. Different amounts of these two solutions were mixed with water for a total volume of 500 μ L, a constant concentration of NBPT (1 mM), and a varying concentration of CD- α (from 50 to 100 mM).

Then, mixtures were freeze-dried and the resulting solid dissolved into 500 μ L of D₂O, before ¹H NMR analysis. For each concentration of CD- α ([CD- α]), and for each proton (*i*) of NBPT, the variation of chemical shift $\Delta\delta_{[\text{CD-}\alpha]}^i$ was calculated thanks to equation 4:

$$\Delta\delta_{[\text{CD-}\alpha]}^i = \delta_{[\text{CD-}\alpha]}^i - \delta_0^i \text{ (Eq. 4)}$$

Then, $1/\Delta\delta_{[\text{CD-}\alpha]}^i$ was plotted against $1/[\text{CD-}\alpha]$. Resulting curves obey to a linear equation. The association constant of the complex (K_a) and the variations of chemical shifts of NBPT in the pure complex ($\Delta\delta_{\text{Cpx}}^i$) were deduced from equations of the intercept and the slope of the curves:

$$\text{intercept} = 1/\Delta\delta_{\text{Cpx}}^i \text{ (Eq. 5) and slope} = 1/K_a \cdot \Delta\delta_{\text{Cpx}}^i \text{ (Eq. 6)}$$

This method was applied on the four signals corresponding to the butyl chain of the NBPT. The four estimations of the association constant were averaged.

2.5. Evaluation of the inhibitory activity

One milliliter of buffer solution (KH₂PO₄/K₂HPO₄, 70 mM, pH 7), 1 mL of inhibitor solution (at different concentrations), and 10 μ L of jack bean urease solution (2.8 mg/mL, Sigma U-1500 35.500) were mixed in test tubes and incubated for 30 min at 37 °C (blank solution consisted of the same mixture without inhibitor). Afterward, 1 mL of urea solution (6 mg/mL) was added, with incubation again for 30 min at 37 °C. The reaction was stopped by the

addition of 2 mL of 0.033 N HCl. Each inhibitor dose was tested in triplicate. The level of ammonium released was determined photometrically, using the ammonium Test from Merck (Ref 1.14752.0001). The inhibition percentage $Inh(\%)$ was calculated as:

$$Inh(\%) = 100 - 100(A_{Inh}/A_B) \text{ (Eq. 7)}$$

where A_{Inh} and A_B are the absorbance at 692 nm in the test tubes, with and without inhibitor, respectively.

3. Results and discussion

Before managing formulations with polysaccharides, a simple method for monitoring degradation of NBPT, solubilized in a solvent, under acidic conditions was developed. While some analysis were already proposed (Abraham, Benson & Jardine, 1983; Douglass & Hendrickson, 1991), NMR data, and more especially ^{31}P NMR in the case of phosphorous-containing compounds, offers the opportunity to also give structural data of resulting products (Fig. 2). In order to mimic acidic soils, this study was therefore performed in an acetate buffer at pH 5.5 over one week. It was firstly observed that the intensity of the initial signal at nearly 60 ppm disappeared in favor of another one at 53 ppm. Secondly, no more NBPT could be detected after one week in this medium. Moreover, the high field chemical shift corresponding to the newly formed compound indicated a break of at least one P-N linkage, substituted by a P-O bond. This result was subsequently complemented and corroborated by a high resolution mass spectrometry (HRMS) analysis (Fig. 2). Indeed, two peaks at m/z 111.56 and 73.87 appeared, thus demonstrating the apparition of diamoniposphoric acid (DATP) and *n*-butylamine, respectively. This showed the favored cleavage between the P atom and the N one connected to the butyl chain, both P-NH₂ linkages remaining stable. Moreover, under the conditions used, no NBPTO was observed. That means that the P=S bond is stable under conditions used and that S/O exchange occurs only in soils, probably thanks to bacterial

activity. Finally, the monitoring of the degradation of NBPT over one week allowed us to show that it followed a first order kinetics at pH 5.5 ($k = 2.2 \times 10^{-6} \pm 0.8 \times 10^{-6} \text{ s}^{-1}$, $n = 15$; See Supplementary materials).

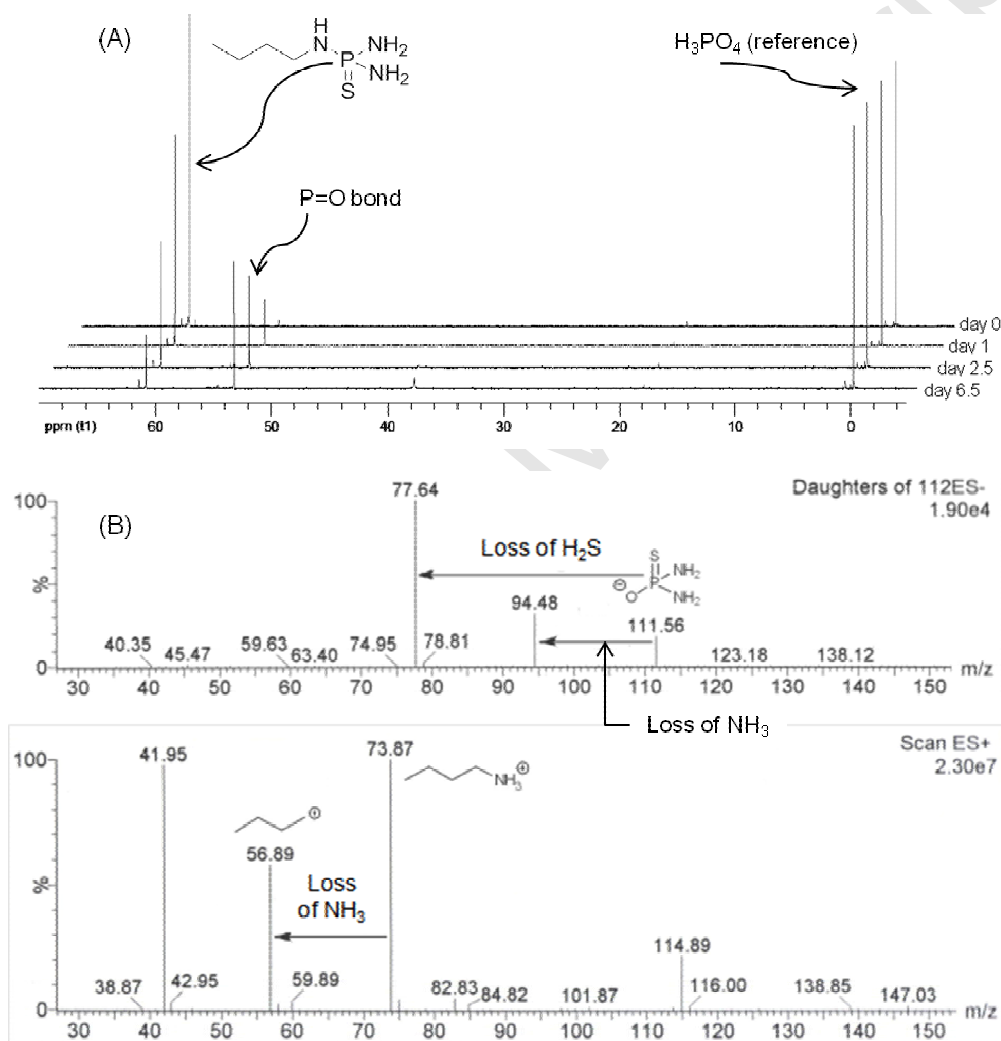


Fig. 2. (A) ^{31}P NMR monitoring and (B) HRMS analysis of the degradation of NBPT at pH 5.5.

213 With this analytical method in hand, it was further planned to build the inhibitor around an
214 environment conducive to its preservation in acidic media. However, NBPT must remain free
215 in order to emerge this background, and be gradually released to exert the desired biological
216 function on urease. On the basis of conformational and physico-chemical adaptability of
217 polysaccharides, we first studied carraghenans (Fig. 3), well known carbohydrates extracted
218 from red seaweeds *Rhodophyceae*. κ - and ι -Carraghenans are characterized by sequence of D-
219 galactopyranosyl (D-Galp) residues having alternatively 4C_1 and 1C_4 conformations, the latter
220 resulting from a 3,6-anhydro-bridge, and they can adopt double strand conformations. λ -
221 Carraghenans are also built of sulfated D-Galp entities, but differ from the latter families by
222 the absence of anhydro-bridge and by the number of anionic groups, thus resulting in tertiary
223 ribbon structure (Cui, 2005). Despite their charges, carraghenans are able to interact with
224 small volatile compounds (Hambleton, Fabra, Debeaufort, Dury-Brun & Voilley, 2009). We
225 thus hypothesized that it could possibly interact either with the butyl chain of NBPT, even
226 weakly, or also with the more polar phosphoramidate part of the inhibitor, or both functions. On
227 this basis, the studied inhibitor was dissolved in saturated solution of κ -, ι - or λ -carraghenans
228 buffered at pH 5.5. Its degradation was monitored by ^{31}P NMR and compared to that of NBPT
229 formulated as Agrotain. Unfortunately, no protecting effect was observed and the
230 disappearance of NBPT in carbohydrate solutions over a period of 6 days followed a kinetic
231 comparable with that of commercially available formulation (See Supplementary Materials).

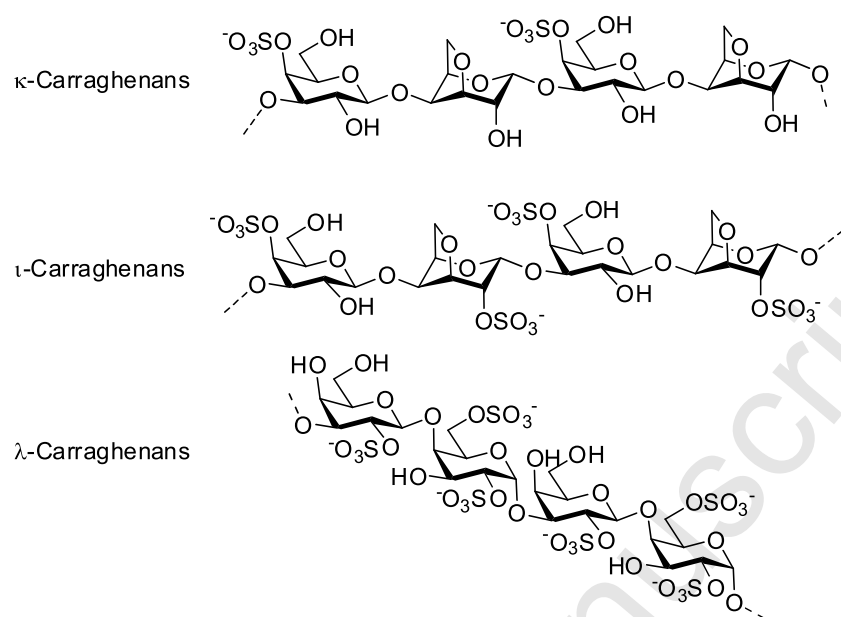


Fig. 3. Primary structures of κ -, ι -, and λ -carraghenans.

In a second stage, alginates were also tried to potentially carry the target thiophosphoramidate. As carraghenans, they present anionic groups, i.e. carboxylates, but not sulfates. We thus anticipated that carboxylate groups in alginates could quench some protons from the media, thus preventing NBPT hydrolysis. Moreover, L-guluronic entities, but not D-mannuronic residues, may form cavities which can accommodate divalent cations such as calcium (Fig. 4). Thus, in the presence of calcium salts, the polyguluronic chains gather around cations, creating a network of channels with some facial amphiphilicity. We have however observed no significant improvement of the formulations, using alginates alone or these polysaccharides in the presence of calcium salts to provide beads. The latter cannot retain efficiently the NBPT molecules since they diffuse into the aqueous solution within only a few minutes.

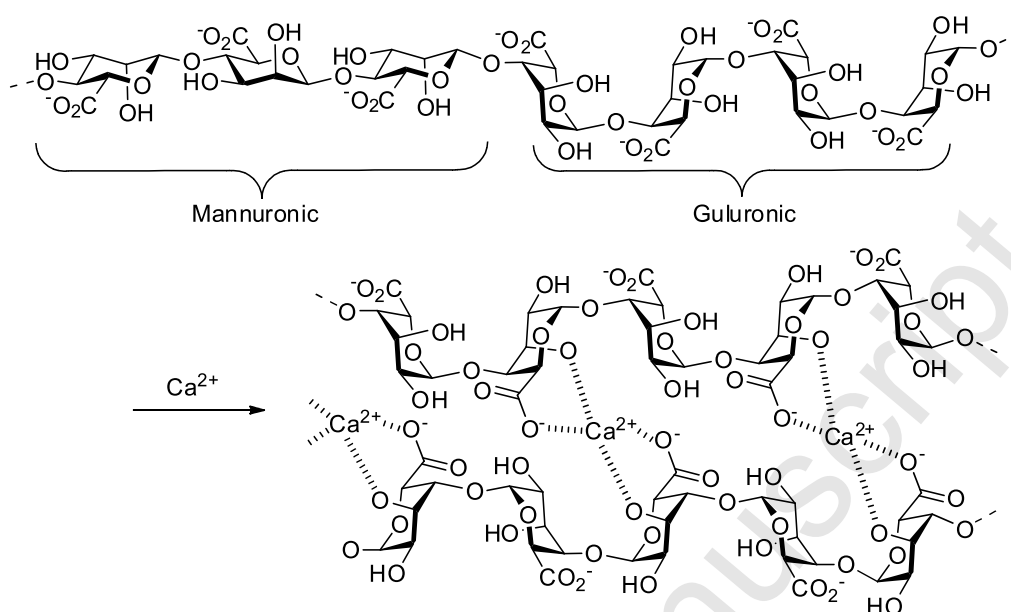


Fig. 4. A conformational model for alginates.

These results led us to turn to neutral polysaccharides. Agar present tertiary conformations close to that of κ - and ι -carraghenans; laminarines, also called β -(1,3)-glucans, can organize in triple helix, and was already used to guide the synthesis of hydrophobic nanomaterials (Lehtovaara & Gu, 2011; Young, Dong & Jacobs, 2000); starch was also selected since it is well established that it can encapsulate iodine, lipids, surfactants by formation of inclusion complexes (Heinemann, Escher & Conde-Petit, 2003) (Fig. 5). Once again, no stabilization was obtained using both agar and laminarines. Nevertheless, a slight improvement was observed using starch previously unstructured in a boiling aqueous solution. Indeed, 60% of NBPT remained unchanged after nine days while Agrotain was decomposed at 60% over the same period. We hypothesize that this positive effect can be assigned to the presence of helices that prefigure maltocyclodextrins.

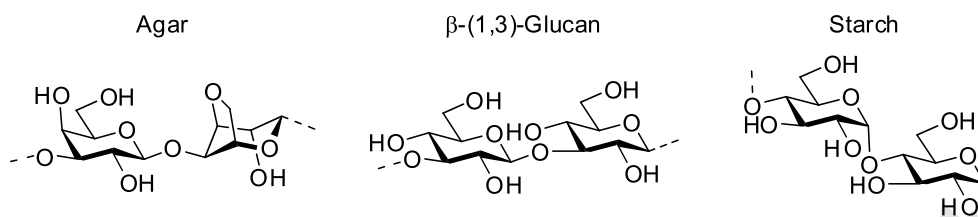


Fig. 5. Main skeleton for agar, β -(1,3)-glucans and starch.

Therefore, this result encouraged us to use pure cyclodextrins instead of polysaccharides, since they are commonly used in the pharmaceutical industry but also in the food and plant health fields (Shen, Yang, Wang, Zhou & Chen, 2012). We expected that the lipophilic cavity of a cyclodextrin interact with the NBPT butyl chain to form a complex able to protect the inhibitor from surrounding acidic medium. It was firstly easily established that the more suitable cyclodextrin likely to favorably interact with NBPT was the smaller one containing six glucosyl residues (α -cyclodextrin, CD- α) (Fig. given in Supplementary Material). Secondly, the optimal molar ratio CD- α /NBPT was experimentally determined as 1:1, and Fig. 6 shows that at least 70% of NBPT remained stable after four days at pH 5.5, while 60% of NBPT from Agrotain formulation was hydrolyzed under the same conditions. This corresponds to an increase of 75% stability

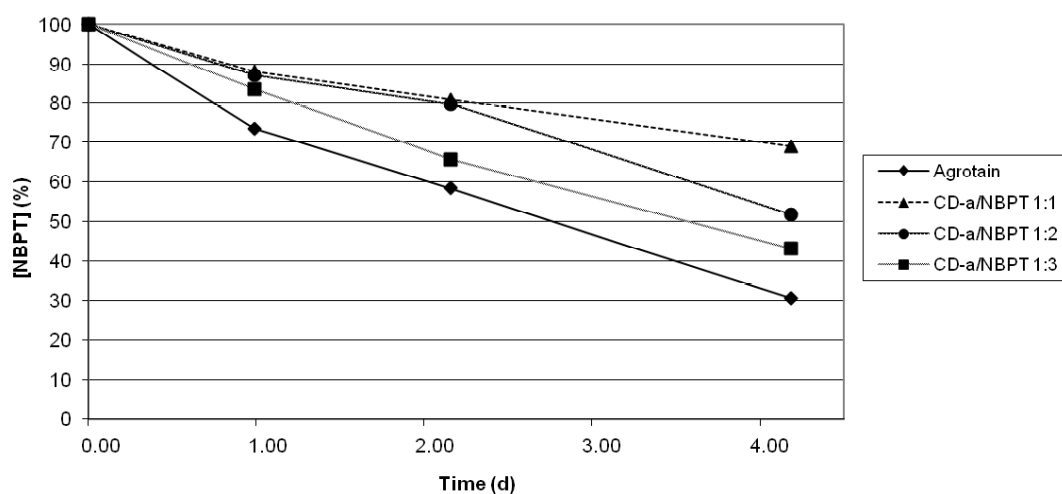


Fig. 6. Stabilization of NBPT according to the molar ratio CD- α /NBPT.

Finally, the precise nature of the suspected complex was determined by ^1H NMR thanks to the Job's plot method (Marçon, Mathiron, Pilard, Lemaire-Hurtel, Dubaele & Djedaini-Pilard, 2009). This approach is based on the correlation between the complex concentration in solution and the chemical shift variations of protons in both the host (CD- α) and the guest (NBPT; see Supplementary Material). More particularly, the complex concentration is proportional to the $\Delta\delta_X \cdot r_X$ where $\Delta\delta_X$ corresponds to the chemical shift variation for proton X and r_X is the ratio $[\text{NBPT}] / ([\text{NBPT}] + [\text{CD-}\alpha])$. Because of low variations observed from protons of CD- α , the Job graphic was drawn from signals corresponding to the butyl chain (Fig. 7). A 1:1 complex was thus unambiguously established.

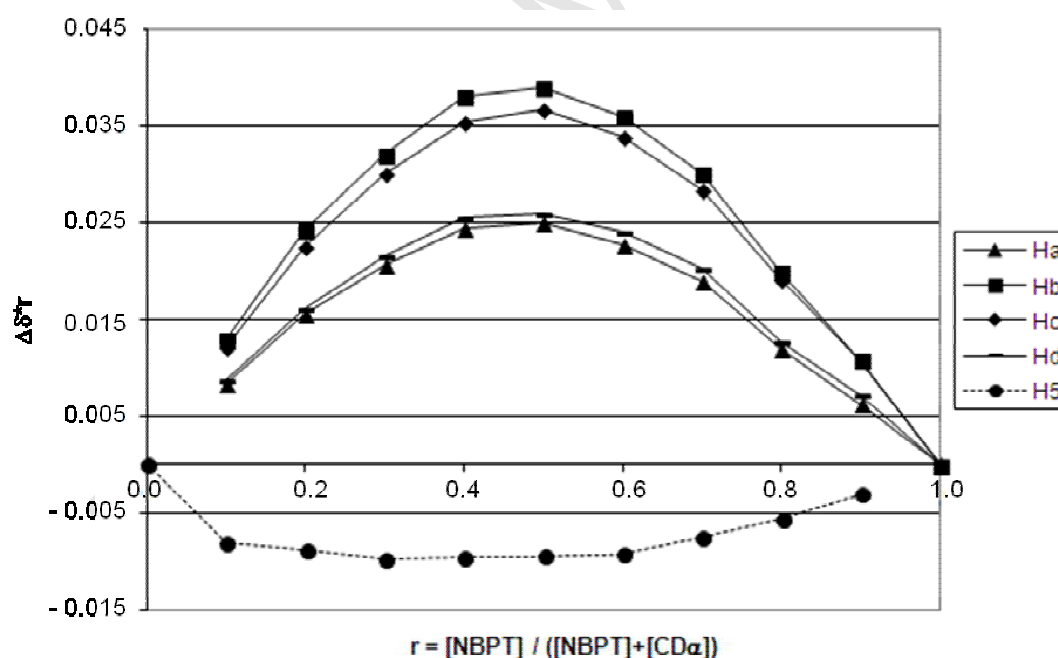


Fig. 7. Determination of the CD α /NBPT according to the Job's plot method (Marçon, Mathiron, Pilard, Lemaire-Hurtel, Dubaele & Djedaini-Pilard, 2009). Ha-Hd are for the alkyl chain of NBPT and H5 for the CD α .

Moreover, this study was complemented by a T-ROESY NMR analysis (Fig. 8) in order to identify precise molecular interactions between the inhibitor and its ligand. The resulting spectrum clearly established that H-5 of the maltocyclodextrin are close to the ethyl terminal group of the alkyl chain of NBPT, and that H-3 of the former, exposed from the bigger crown, could interact with all protons of the butyl chain, but more particularly with the core CH₂.

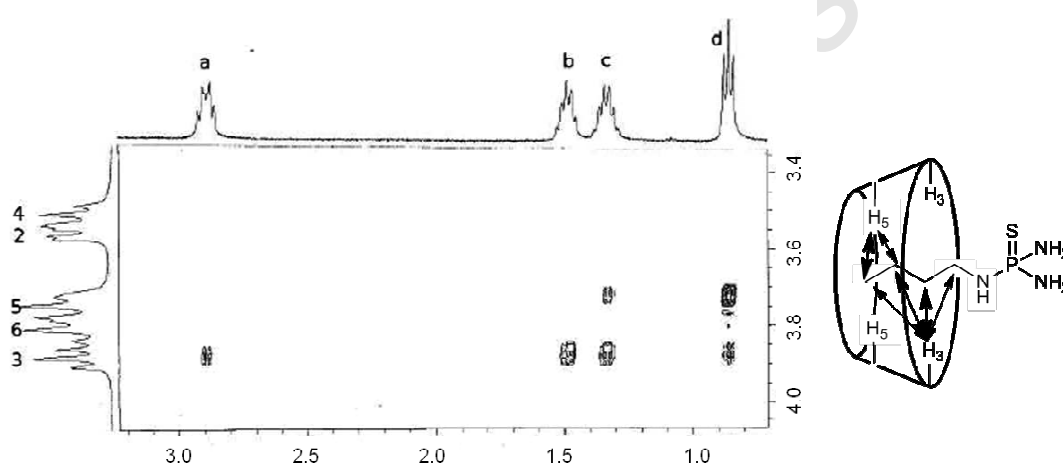


Fig. 8. Focus on NRM T-ROESY spectrum of the inclusion complex CD α /NBPT.

Finally, in order to characterize the dynamic of the equilibrium, the association constant K_a was determined according to the Benesi-Hildebrand method, applied on ¹H NMR signals corresponding to the butyl chain of the NBPT (Benesi & Hildebrand, 1949). Using a large excess of CD- α and a constant concentration in NBPT, all resulting straights corresponding to the protons of NBPT gave a value of $94 \pm 4 \text{ M}^{-1}$, slightly in favor of the formation of the complex.

Having these physicochemical data in hands, the inhibitory activity of the established more stable complex was evaluated *in vitro* at pH 7, under optimal conditions for the activity of Jack bean urease. A value of 1.5 μ M was obtained, i.e. 15 times higher than the IC₅₀ of free NBPT (100 nM). As expected, ligation of NBPT by the α -cyclodextrin was accompanied by a significant decrease of inhibiting property, but this lower activity has to be put into perspective with increased stability over time at pH 5.5 and with the significant simplicity of the formulation. To conclude this study, the 1:1 inclusion complex as well as solutions of CD- α /NBPT with excess cyclodextrin (ratio: 2:1 and 5:1) were deposited on an acidic soil. Monitoring of the urea concentration showed that this simple new formulation had similar efficiency than that of Agrotain (See Supplementary Material). Since activity *in vitro* was more interesting for the complex, limitations could be attributed to the bacterial activity from the soil.

4. Conclusion

Our study is part of the current trend to formulate bioactive compounds, especially in the field of agriculture, as simple as possible using a limiting number of additives, obviously environmentally friendly. In this context, a new formulation of NBPT, a urease inhibitor widely used as fertilizer, was searched with the main goal of increasing its stability over time under acidic conditions. Our approach was based on molecular characteristics of this thiophosphotriamide since it presents a polar phosphoramidate function and a lipophilic butyl chain. Therefore, a panel of polysaccharides, neutral or charged, with different physicochemical properties, was tested. Nevertheless, the best results were obtained with the α -cyclodextrin used as additive. The 1:1 inclusion complex was fully characterized and presented moderate inhibition activity (1.5 μ M) but a significant increased stability at pH 5.5 compared to that of commercial Agrotain (75% after 4 days). The resulting inclusion complex

is a stable solid which is highly hydrosoluble. Application of solutions in an acidic soil finally showed that inclusion complex of NBPT in cyclodextrin- α had similar inhibition efficiency than Agrotain.

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Captions

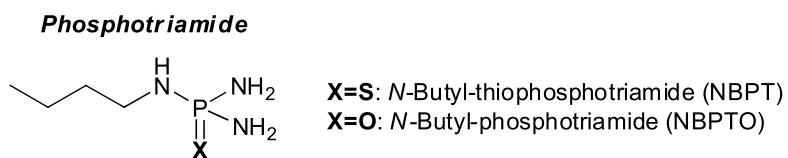


Fig.1. Structure of the studied urease inhibitor NBPT.

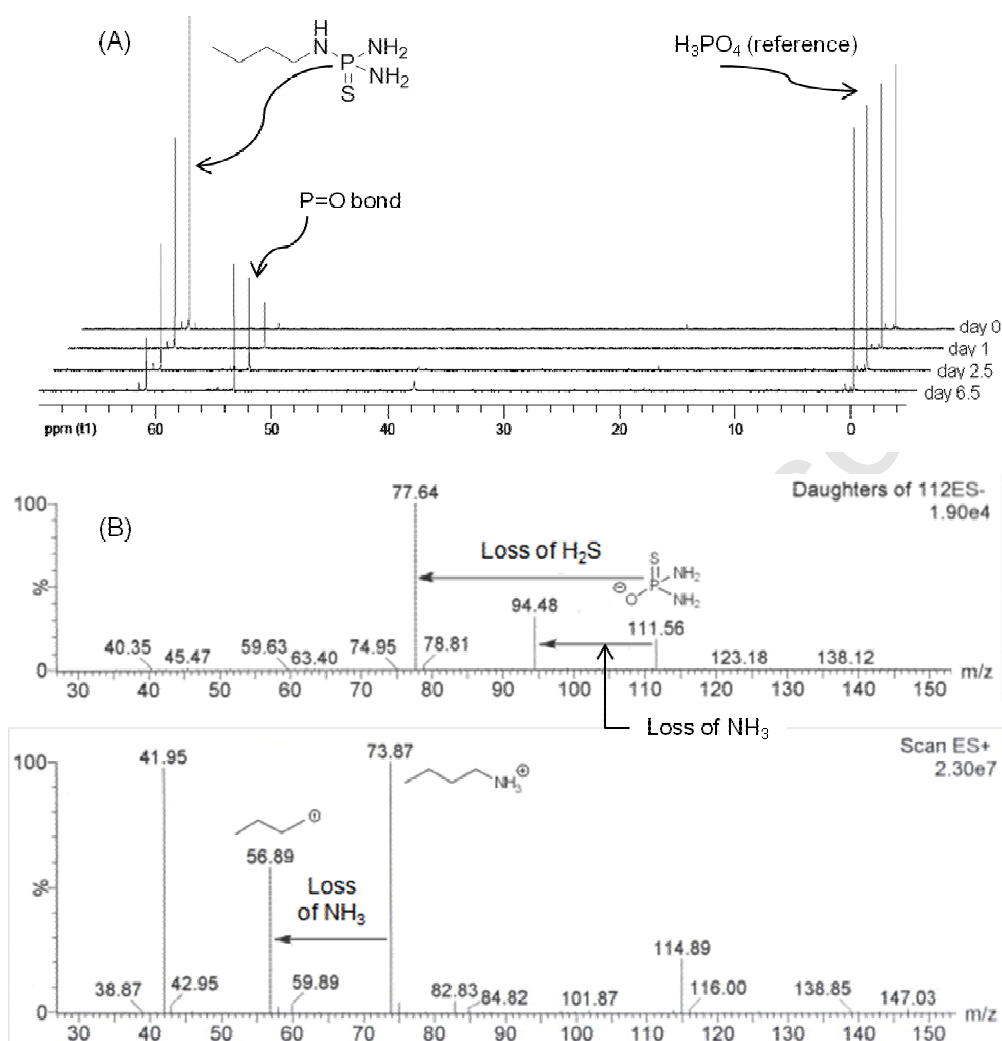


Fig. 2. (A) ^{31}P NMR monitoring and (B) High Resolution Mass Spectrometry analysis of the degradation of NBPT at pH 5.5.

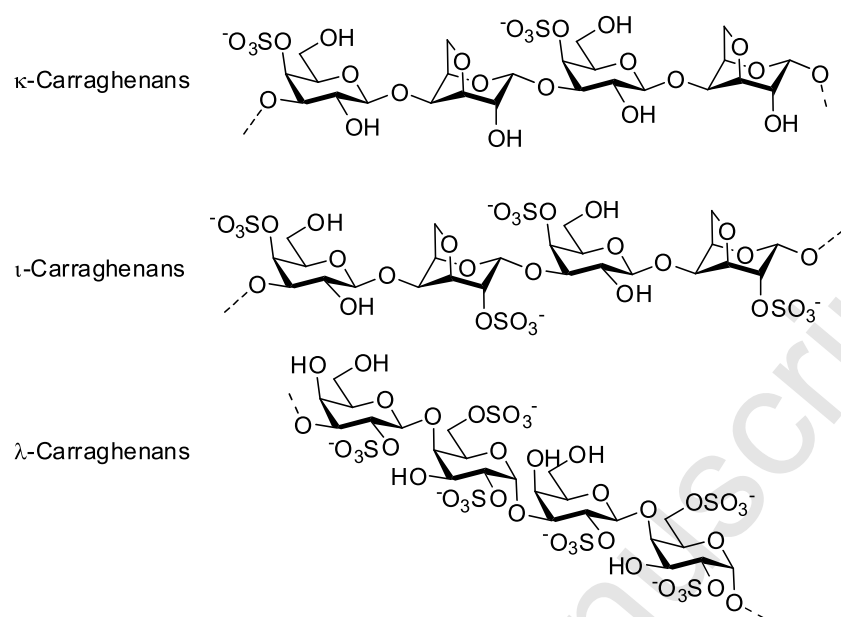


Fig. 3. Primary structures of κ -, ι -, and λ -carraghenans.

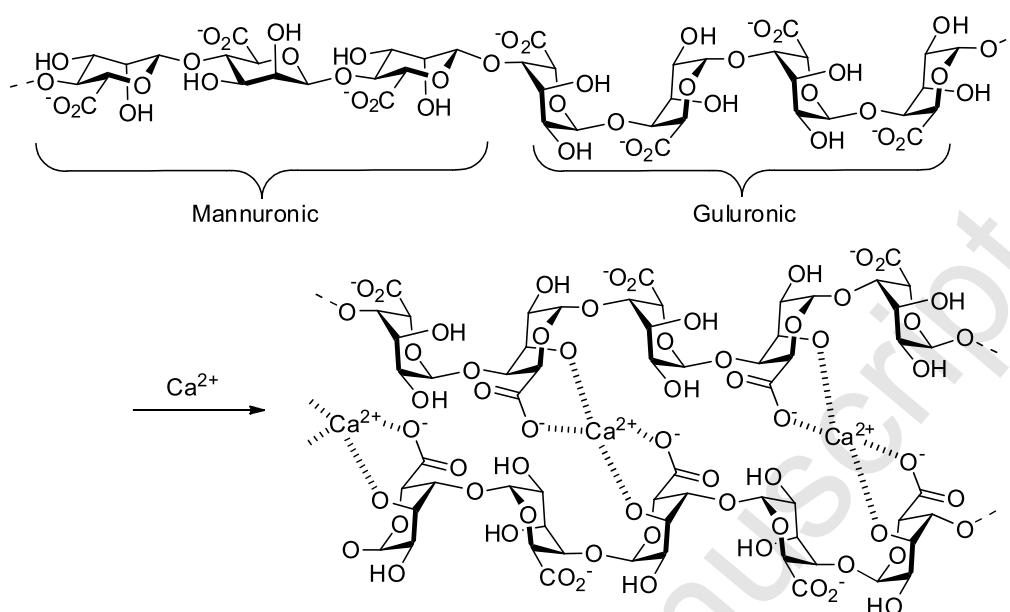


Fig. 4. A conformational model for alginates.

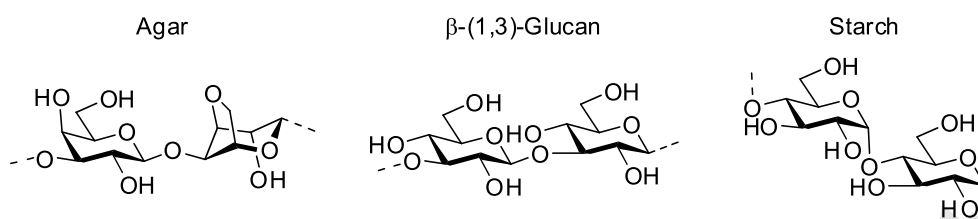


Fig. 5. Main skeleton for agar, β -(1,3)-glucans and starch.

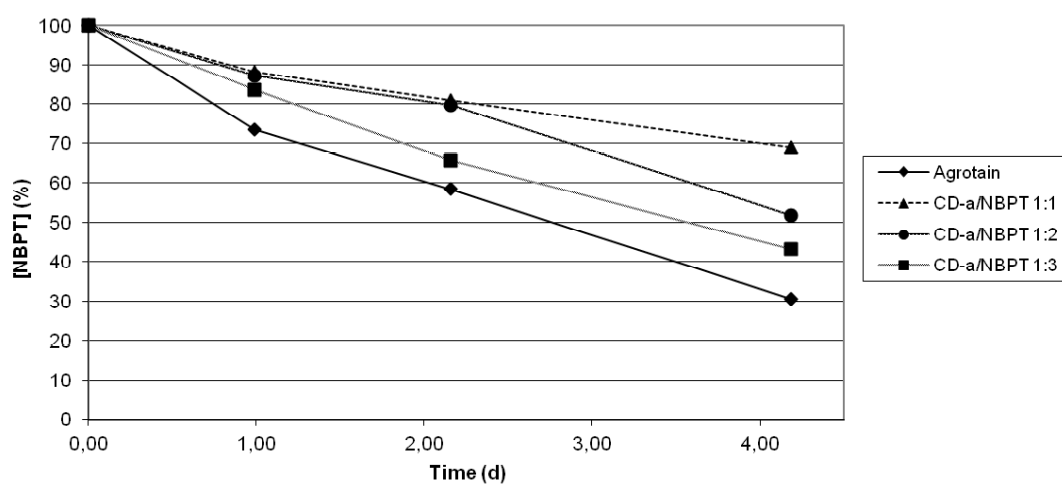


Fig. 6. Stabilization of NBPT according to the molar ratio CD- α /NBPT.

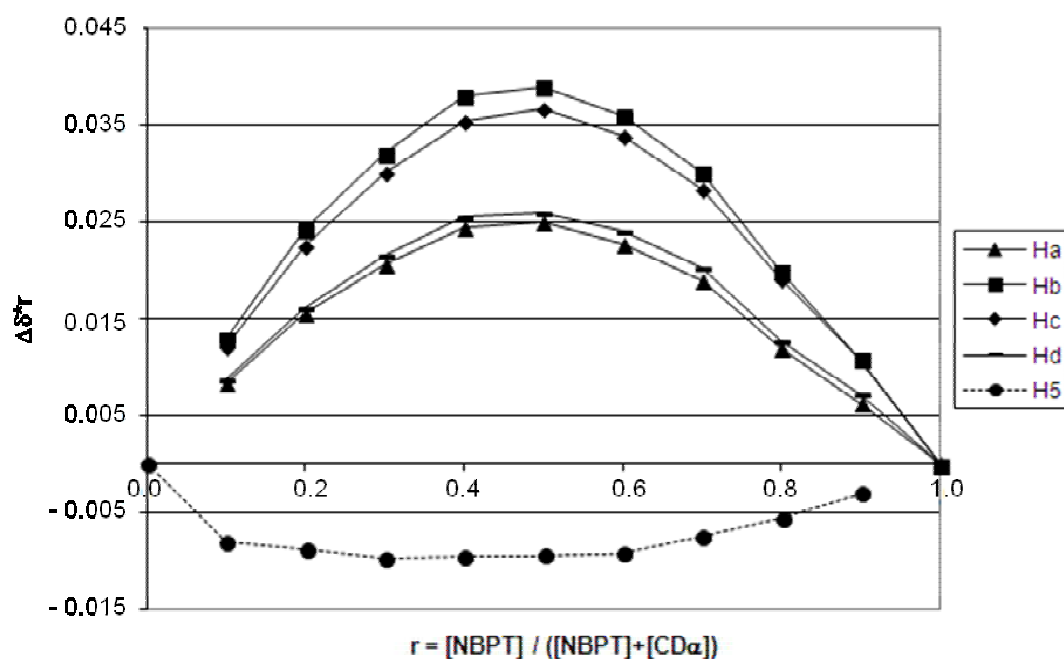


Fig. 7. Determination of the CDα/NBPT according to the Job's plot method (Marçon, Mathiron, Pilard, Lemaire-Hurtel, Dubaele & Djedaini-Pilard, 2009). Ha-Hd are for the alkyl chain of NBPT and H5 for the CDα.

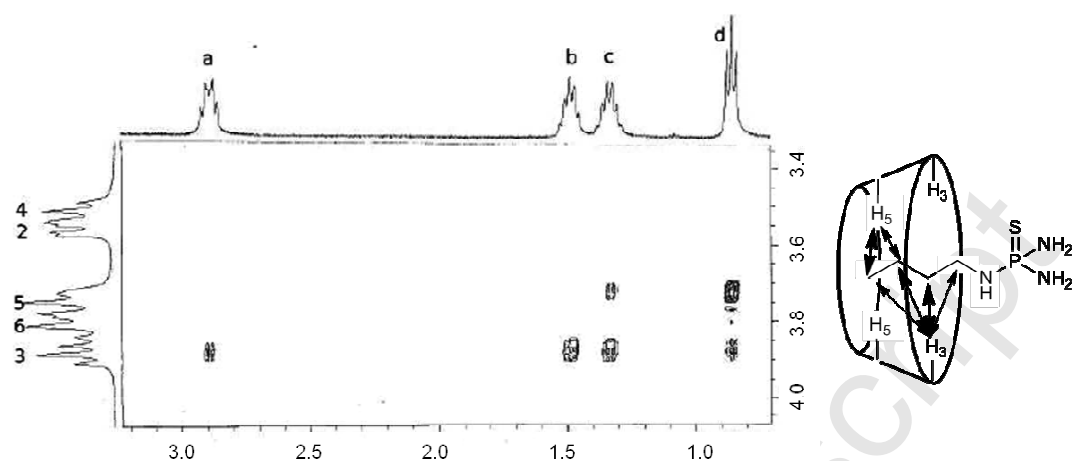


Fig. 8. Focus on NRM T-ROESY spectrum of the inclusion complex CDα/Nzz